Influence of *Euonymus alatus Sied* extracts on MDCK proliferation and high concentration of glucose induced cell injury

Haiyan Yang[1,*], Qiujuan Wang[2], Danni Zhu[2]

[1]*Institute of Food and Drug Control of Henan Province, Zhengzhou, Henan* 450003*, China;* [2]*China Pharmaceutical University, Nanjing, Jiangsu* 210009, *China*

Received September 12, 2008

Abstract

Objective. To study the effects of extracts (SML-1, SML-2, SML-3 and SML-4) from *Euonymus alatus Sied* on cell proliferation and on renal tubular cell (Madin-Darby Canine Kidney, MDCK) injury induced by high concentration of glucose. *Methods.* MTT assay was used to evaluate the effects of herbal extracts on cell proliferation and viability in MDCK cells after exposure to high concentration of glucose. Lactate dehydrogenase (LDH), N-acetyl-β-amino glucokenase (NAG) and alkaline phosphatase (ALP) in the culture medium were determined by enzyme-linked kits assay. *Results.* MTT results showed the cell inhibitory rates in drug groups were significantly lower than that of the positive control (cisplain) in normal MDCK cells except SML-1 (100 μg/ml) and SML-2 (100 μg/ml). Following high concentration of glucose exposure there was a significant rise in LDH release rate and NAG concentration, and a drop in ALP concentration. The extracts were proved to be effective in reducing these glucose-induced injurys in MDCK cells. *Conclusion.* Along with high concentration of glucose, extracts from *Euonymus alatus Sied* may be useful therapeutic agents in treating renal tubular cell injury. Our findings also support that MDCK were sensitive to high concentration of glucose and the changes of cell permeability and antioxidant capacity induced by high concentration of glucose might be linked to the pathogenesis of diabetic nephropathy. [Life Science Journal. 2008; 5(4): 41 – 46] (ISSN: 1097 – 8135).

Keywords: *Euonymus alatus Sied*; MDCK; cell proliferation; high concentration of glucose

1 Introduction

Proximal tubular epithelial cells are the most abundant cells in the renal cortex, and recent studies suggest that they may play an important role in initiating pathological changes in renal disease. Concentration changes of interstitial glucose influence the progression of interstitial fibrosis. Na-glucose co-transporter and glucose transporter-1 transport glucose into cells leading to high glucose concentration inside the cells[1]. Hyperglycemia is directly involved in the development of diabetic nephropathy (DN). In the early period of diabetic nephropathy, volumetric increment of kidney that mainly caused by pathological changes of glomerulus, renal tubule and interstitial tissues may occur. So the pathological changes in renal tubule and interstitial tissues have very important effects on hypertrophy of kidney[2].

In our study, Madin-Darby Canine Kidney (MDCK) cells were used to observe the effects of extracts (SML-1, SML-2, SML-3, and SML-4) from *Euonymus alatus Sied* on cell proliferation and cell viability and the enzymatic activities of LDH, NAG and ALP in renal tubular cells on exposure to high concentration of glucose.

2 Materials and Methods

2.1 Herbal extracts

Herbal extracts (SML-1, SML-2, SML-3 and SML-4) from *Euonymus alatus Sied* were provided by Institute of Chinese Traditional Medicine of China Pharmaceutical University, and the extracts were prepared through lixiviation, purification, condensation and exsiccation. SML-1 was extracted from ethyl acetate; SML-2 was extracted from 50% alcohol; SML-3 was extracted from 3, 4-dihydrobenzoic acid; and SML-4 was extracted from water.

*Corresponding author. Email: qjwangnj@sina.com.cn

2.2 Cell cluture

MDCK provided by New Drug Screening Center in China Pharmaceutical University, were cultured as described before[3]. In brief, cells were cultured under standard conditions in RPMI 1640 (Gibco, USA) supplemented with 10% fetal calf serum (PAA, Austria) in a humidified atmosphere of 5% CO_2 / 95% air at 37 ºC. After 60% – 80% confluence, the cells were trypsinized with 0.25% trypsin (AMRESCO, USA, dissolved in PBS, pH 7.4), counted and passaged.

2.3 MTT assay

Cell proliferation was determined based on previously described method[4,5]. The MDCK cells in exponential growth were inoculated into two 96-well plates (2×10^4 cells/well). Medium was changed after 24 hours. Cells of the blank control group were cultured in RPMI 1640 medium (10% FBS) only. In one plate, the cells were treated with extracts at different final concentrations (100, 50, 25, 12.5, 1.0, 0.1, 0.01 μg/ml). In another plate, cells were treated with glucose (30 mM) combined with extracts at different final concentrations (10, 1.0, 0.1, 0.01 μg/ml). 20 μl of MTT (Sigma, USA) was then added at 48 hours after treatments. Four hours later, the supernatant was discarded and 150 μl of DMSO was added. The optical density of each well was measured at λ_{570nm} by microplate reader (Bio-Rad, USA). Cisplatin was used as the positive control because it inhibits the function of renal tubule lysosome, destroy brush border and restrain cell proliferation according to morphological researches[6].

The inhibitory rate (%) = (mean OD value of blank control group – OD value of treated group) ×100 / mean OD value of blank control group.

2.4 Assay of LDH release rate

The LDH release rate was performed as previously described[7,8]. Briefly, the MDCK cells were subcultured in a 24-well plate at a density of 10^5 cells/ml. After 80% confluency, medium was changed into RPMI 1640 medium with 2% FBS. After 24-hour culture, the cells were exposed to glucose (30 mM) and extracts (5, 0.5 μg/ml) for additional 24 hours. Supernatant of each well was stored in – 80 ºC for the measurement of extracellular LDH (Us). Cells were washed with ice-cold PBS and broken up for the measurement of intracellular concentration of LDH (Uc). LDH was a stable cytoplasmic enzyme which was present in cells. When the plasma membrane was damaged, LDH was rapidly released out of cell. LDH activity was determined at the wavelength of 440 nm.

LDH release rate (%) = Us × 100 / (Uc + Us)

2.5 Measurement of NAG and ALP activity

The activities of NAG and ALP were measured as previously described method[8,9]. The assay kits were from Jiancheng Bioengineering Institute (Nanjing, China) Briefly, the MDCK cells were subcultured in a 12-well plate at a density of 2×10^5 cells/ml. 24 hours later mediums were changed into RPMI 1640 with 2% FBS. After 24 hours treatment with glucose (30 mM) and extracts (5, 0.5 μg/ml), supernatant of each well was used for the measurement of NAG and ALP at 400 nm and 520 nm respectively.

2.6 Statistical analysis

The results were expressed as $\bar{X} \pm$ SD. The statistical analysis involving two groups was performed by means of Student's *t*-test. All data were processed with SPSS 14.0 software. $P < 0.05$ was considered significant, and $P < 0.01$ was considered more significant.

3 Results

3.1 Cell proliferation of MDCK

As shown in Table 1 and Figure 1, SML-1 (100 μg/ml) and SML-2 (100 μg/ml) showed the cytotoxic effect on MDCK at 48 hours after treatment (64.7%, 70.8%), whereas the growth inhibitory rate of cisplatin was 68.2%. In contrast to cisplatin, extracts SML-1 (1 – 50 μg/ml), SML-2, SML-3, and SML-4 didn't show evident cytotoxic effect on normal MDCK cells.

MTT assay was used as an indirect measurement to determine the viability of MDCK exposed to the glucose (30 mM). The effects of the four extracts were also revealed in Table 2 and Figure 2. After incubation with glucose for 48 hours, the MDCK cells death reached maximal (21.7%). SML-3 (1 μg/ml), SML-4 (10, 1, 0.1, 0.01 μg/ml) was considered to be more effective against the cytotoxicity of glucose on MDCK cell proliferation.

3.2 LDH release rate in high concentration of glucose induced MDCK

The results presented in Table 3 showed that after treatment for 24 hours, the permeability of cells were changed by glucose (30 mM) and enzyme leaked out, which indicated plasma membrane was damaged. The plasma membrane damage induced by high concentration of glucose were decreased by the four extracts as compared with the model group, which perhaps due to the lower LDH release rate (Figure 3).

Table 1. Normal MDCK proliferation with extracts from *Euonymus alatus Sied* ($\bar{X}$ ± SD)

Group	Concentration (μg/ml)	Inhibitory rate (%)
Blank control	–	0.0
Cisplatin	10	68.2$^{\#\#}$
SML-1	100	64.7
	50	4.3**
	25	11.7**
	12.5	12.2**
	1	13.9**
	0.1	7.1**
SML-2	100	70.8
	50	12.6**
	25	13.9**
	12.5	– 18.8**
	1	– 9.6**
	0.1	3.9**
SML-3	100	6.5**
	50	4.1**
	25	18.0**
	12.5	4.1**
	1	– 9.5**
	0.1	– 23.5**
SML-4	100	– 5.7**
	50	0.4**
	25	5.5**
	12.5	– 1.2**
	1	– 2.1**
	0.1	– 24.7**

$^{\#\#}$: *vs.* blank control, $P < 0.01$; **: *vs.* cisplatin, $P < 0.01$. $n = 8$.

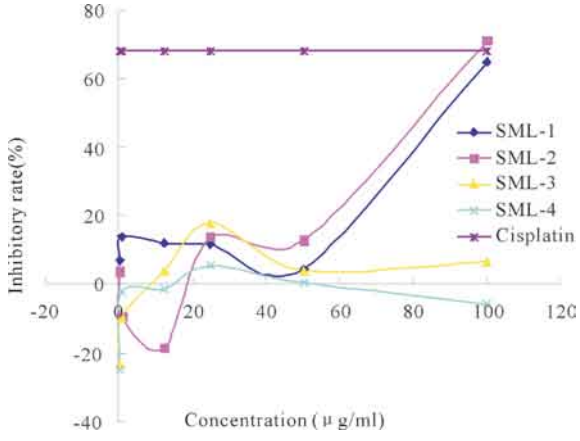

Figure 1. Effect of SML on cell inhibitory rate in normal MDCK.

Table 2. High concentration of glucose-induced MDCK proliferation with extracts from *Euonymus alatus Sied* ($\bar{X}$ ± SD)

Group	Concentration (μg/ml)	Inhibitory rate (%)
Blank control	–	0.0
Glucose (30 μM)	–	21.7$^{\#\#}$
SML-1	10	17.3
	1	17.8
	0.1	17.6
	0.01	22.2
SML-2	10	34.9
	1	24.4
	0.1	18.5
	0.01	28.8
SML-3	10	24.6
	1	13.7
	0.1	20.7
	0.01	24.1
SML-4	10	13.6*
	1	8.6**
	0.1	15.2*
	0.01	16.6*

$^{\#\#}$: *vs.* blank control, $P < 0.01$; *: *vs.* glucose, $P < 0.05$, **: $P < 0.01$. $n = 8$.

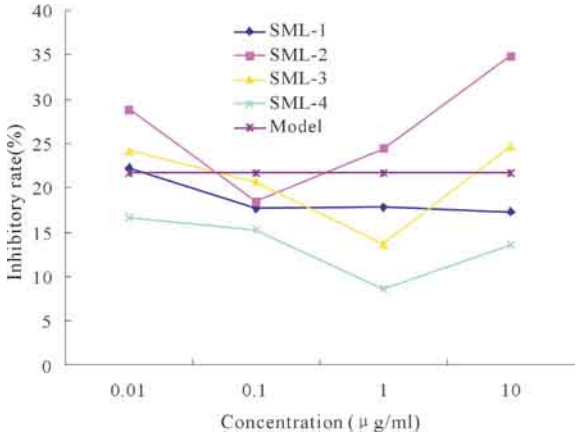

Figure 2. Effect of SML on cell inhibitory rate in high concentration of glucose-induced MDCK.

3.3 NAG in high concentration of glucose induced MDCK

The results in Table 4 & Figure 4 demonstrated that lysosome injury was induced by high concentration of

Table 3. LDH release rate in high concentration of glucose induced MDCK ($\bar{X}$ ± SD)

Group	Concentration (μg/ml)	LDH releasing rate (%)
Blank control	–	14.57 ± 1.55
Glucose (30 μM)	–	19.71 ± 1.29##
SML-1	5	19.48 ± 1.31
	0.5	15.18 ± 0.81**
SML-2	5	17.74 ± 1.45
	0.5	17.16 ± 2.64
SML-3	5	13.39 ± 1.00**
	0.5	14.55 ± 0.76**
SML-4	5	17.77 ± 4.13
	0.5	11.97 ± 1.94**

##: *vs.* blank control, $P < 0.01$; **: *vs*. glucose, $P < 0.01$. $n = 6$.

Table 4. NAG activity in high concentration of glucose induced MDCK ($\bar{X}$ ± SD)

Group	Concentration (μg/ml)	NAG (U/g prot)
Blank control	–	17.38 ± 1.60
Glucose (30 μM)	–	28.85 ± 1.99##
SML-1	5	26.64 ± 2.58
	0.5	22.04 ± 4.74*
SML-2	5	18.92 ± 2.37**
	0.5	17.58 ± 0.54**
SML-3	5	26.61 ± 0.51
	0.5	19.63 ± 3.36*
SML-4	5	21.70 ± 0.14*
	0.5	24.31 ± 1.88*

##: *vs.* blank control, $P < 0.01$; *: *vs*. glucose, $P < 0.05$, **: $P < 0.01$. $n = 6$.

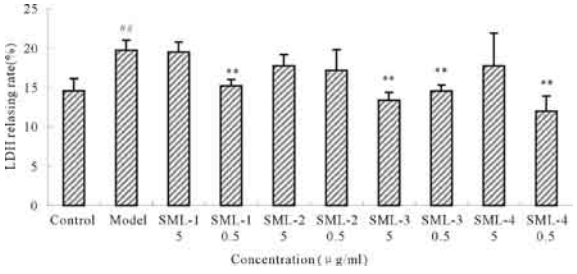

Figure 3. Effect of SML on LDH releasing rate in high glucose-induced MDCK ($\bar{X}$ ± SD, $n = 6$). ##: $P < 0.01$ *vs.* blank control; **: $P < 0.01$, *vs.* glucose.

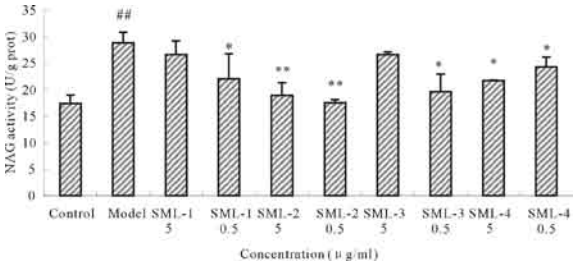

Figure 4. Effect of SML on NAG activity in high glucose-induced MDCK ($\bar{X}$ ± SD, $n = 6$). ##: $P < 0.01$ *vs.* blank control; *: $P < 0.05$, **: $P < 0.01$, *vs.* glucose.

glucose and extracelluar concentration of lysosomal enzyme increased. The strongest protective effects were exerted by the four extracts after incubation for 24 hours in a concentration-dependent manner.

3.4 ALP in high concentration of glucose induced MDCK

To further explore the cytotoxic mechanism of MDCK cells induced by high concentration of glucose, enzyme analysis was carried out to examine the extracellular concentration of ALP. After treatment with glucose (30 mM) for 24 hours, activity of ALP decreased significantly. The results in Table 5 & Figure 5 showed that the four extracts were considered to be effective to improve the cell injury induced by high concentration of glucose.

4 Discussion

For diabetes, pathological changes such as hyperglycemia, changes of hemodynamics had direct influence on the function of epithelial cells and the structures of interstitial in renal tubule. Interstitial changes provoked or aggravated lesion of renal tubule and vascular[10]. In recent years, researches found that tubulointerstitial injury was a major feature of diabetic nephropathy and an important index of renal dysfunction[11].

It has been suggested that the renal tubular cell damage induced by glucose may be involved in the development of DN[12]. In our study, we found that MDCK cells were sensitive to glucose (30 mM) and the changes of cell permeability and antioxidant capacity induced by glucose might be linked to the pathogenesis of DN.

Cell proliferation test were carried out in normal and high concentration of glucose treated MDCK cells. We found that cell proliferation was inhibited after exposure to glucose. According to Ziyadeh[13], cell proliferation-was inhibited and cell hypertrophy occurred under high glucose. Our findings were similar to that of Ziyadeh's.

Table 5. ALP activity in high glucose-induced MDCK ($\bar{X}$ ± SD)

Group	Concentration (μg/ml)	ALP (U/g prot)
Blank control	–	38.87 ± 1.55
Glucose (30 μM)	–	25.09 ± 1.92##
SML-1	5	28.20 ± 0.24
	0.5	31.27 ± 2.39*
SML-2	5	29.64 ± 2.37**
	0.5	30.55 ± 2.48*
SML-3	5	34.98 ± 0.84**
	0.5	26.21 ± 4.19
SML-4	5	26.30 ± 3.53
	0.5	32.63 ± 3.20*

##: *vs.* blank control, $P < 0.01$; *: *vs*. glucose, $P < 0.05$, **: $P < 0.01$. $n = 6$.

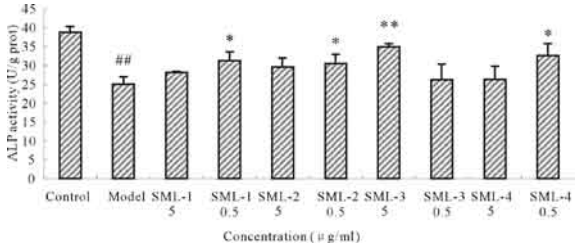

Figure 5. Effect of SML on ALP activity in high glucose-induced MDCK. $\bar{X}$ ± SD, $n = 6$. #: $P < 0.05$, ##: $P < 0.01$ *vs.* blank control; *: $P < 0.05$, **: $P < 0.01$, *vs.* glucose.

We investigated the cytotoxic effects of the four extracts (SML-1, SML-2, SML-3, and SML-4) from Euonymus alatus Sied on MDCK cells and the possible mechanism. SML-3 and SML-4 didn't present any significant inhibitory effects on MDCK cells growth. SML-1 and SML-2 also didn't reveal strong anti-proliferation activity except at the concentration of 100μg/mL.

In our study, we found that the extracts exhibited strong protective effects on MDCK cells treated with glucose (30 mM). Internal release of LDH reflected the viability of cells and LDH participates in the reaction of pyruvate changing into lactic acid. SML-3 was proved to be effective in maintaining cell integrity because of the decreased LDH release rate. Activities of SML-1 and SML-4 were less potent than that of SML-3. SML-2 didn't show any significant protective effects.

Lysosome injury was the primary and sensitive index in renal tubular cells in the diabetics. NAG was considered as the specific enzyme among numerous enzymes in lysosome because its content was abundant in renal tissues[14]. We found that lysosomal enzyme leakage may occurr in MDCK cells on exposure to high concentration of glucose. The activity of NAG decreased and lysosome membrane was stabilized after treated with SML-2 and SML-4. At the concentration of 0.5 μg/ml, SML-1 and SML-3 stabilized the lysosomal membrane.

In this study, the extracts were also shown to increase the activity of ALP. ALP was a characteristic enzyme of brush border[15]. Damaged brush border lead to the failure of enzyme activity in a high glucose surroundings. SML-1, SML-2, SML-3 and SML-4 were found to possess potent protective effects on the high concentration of glucose induced MDCK cells.

5 Conclusion

During the pathogenic progression of diabetes, cell's integrity, lysosome and brush border villus may be injured by the high concentration of glucose in renal tubular cells. Our findings indicated that extracts (SML-1, SML-2, SML-3, and SML-4) from *Euonymus alatus Sied* may be useful therapeutic agents to attenuate renal tubular cell injury in DN. Further studies need to be carried out in our future studies.

References

1. Phillips AO, Sreadman R, Morrisey K, *et al*. Polarity of stimulation and secretion of transforming growth factor-beta 1 by cultured proximal tubular cells. Am J Pathol, 1997; 150: 1101 – 11.
2. Tang Z, Li LS. Pathological, physiological and danger factor of diabetic nephroathy. Chinese Journal of Nephrology Dialysis & Transplantation 1998; 2: 70 – 2.
3. Zimmerhackl LB, Mesa H, Kraemer F, *et al*. Tubular toxicity of cyclosporine A and the influence of endothelin-1 in renal cell culture models (LLC-PK1 and MDCK). Pediatr Nephrol 1997; 11: 778 – 3.
4. Li L, Lau BHS. Protection of vascular endothelial cells from hydrogen peroxide-induced oxidant injury by pyenosides saponins of gynostemma pentaphyllum. Phytotherapy Research 1993; 7: 299 – 307.
5. Niu M, Chang M, Liu SX. Influence on cell proliferation of renal tubule cells under high glucose condition *in vitro*. Liaoning Journal of Practical Diabetology 2003; 11(2): 22 – 3.
6. Leibbrandt MEI, Wolfgang GHI, Metz AL, *et al*. Critical subcellular targets of cisplatin and related platinum analogs in rat renal proximal tubule cells. Kidney Int 1995; 48(3): 761 – 70.
7. Liu SJ, Zhou SW. Influence on enzyme activity of cispaltin in primary cultured rabbit proximate renal tubule cells and protection of Taurine, Sanchi saponin and capillarin. Chinese Pharmacological Bulletin 1999; 15(5): 452 – 5.
8. Li R, Ai GP, Xu H. Toxic effects of depleted uranium on human renal tubule cells. Acta Academiae Medicinae Militaris Tertiae 2004; 26(3): 185 – 8.
9. Wang XQ, Bai XT, Yin XR. Influence of plumbum and chromium on N-Acetyl-β-glucosaminidase in rat renal tubule cells. Journal of Environment and Health 2002; 19(4): 306 – 8.
10. Bohle A, Wehrmann M, Bogenschutz O, *et al*. The pathogenesis of chronic renal failure in diabetic nephropathy: Investigation of 488 cases of diabetic glomerulosclerosis. Pathol Res Pract 1991; 187(2–3): 251

– 9.
11. Gilbert RE, Cooper ME. The tubulointerstitiumin progressive diabetic kidney disease: More than an aftermath of glomerular injury? Kidney Int 1999; 56(5): 1627 – 37.
12. Ziyadeh FN. Significance of tubulointerstital changes in diabetes renal disease. Kidney Int Suppl 1996; 54 (Suppl): S10 – 3.
13. Ziyadeh FN, Goldfarb S. The renal tubulointerstitium in diabetes mellitus. Kidney Int 1991; 39(3): 464 – 75.
14. Lockwood TD, Bosmann HB. The use of urinary N-acetyl-β-glucosaminidase in human renal toxicology. Toxicol Appl Pharmacol 1979; 49(2): 323 – 36.
15. Iaina A, Schwartz D. Renal tubular cellular and molecular events in cute renal failture. Nephron 1994; 68(4): 415 – 8.